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Changes in bacterial loads, gas composition, volatile organic compounds, and glucosinolates of fresh bagged Ready-To-Eat rocket under different shelf life treatment scenarios

Hanis Nadia Yahya[a], Stella Lignou[b], Carol Wagstaff[b], Luke Bell[c,*]

[a] Faculty of Science & Technology, Universiti Sains Islam Malaysia, Bandar Baru Nilai, 71800, Nilai, Negeri Sembilan, Malaysia
[b] Department of Food & Nutritional Sciences, University of Reading, Whiteknights, Reading, Berkshire, RG6 6AP, UK
[c] School of Agriculture Policy & Development, University of Reading, Whiteknights, Reading, Berkshire, RG6 6AP, UK

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ABSTRACT

Temperature abuse and improper shelf life treatment of Ready-to-Eat wild rocket is a leading cause of product quality losses and consumer rejection. It can cause the deterioration of appearance, the build-up of bacterial numbers, and the production of off-odours. This study subjected commercially produced and processed bags of wild rocket to various temperature and shelf life duration treatments that could hypothetically be experienced by a consumer, purchasing bags from supermarkets that have suffered cold-chain breaches. We take a unique experimental perspective that accounts for potential temperature variation scenarios within supermarkets and the home. Bacterial counts, volatile organic chemical production, glucosinolate concentration, and internal bag atmosphere composition were measured under these temperature scenarios and across growing seasons. Our results showed that the season of purchase significantly affects wild rocket respiration and bacterial loads. Prolonged high temperature abuses increased microbial loads, disulfide abundance, and reduced content of the glucosinolate glucoerucin. Short temperature abuse treatments resulted in no significant changes in bacterial numbers, providing bags were returned to cool-chain conditions. Samples stored under these conditions ($< 5\,^{\circ}C$) saw no significant changes. The effect of growth season also significantly affects wild rocket respiration. Summer-grown produce had high bacterial loads, but winter-grown had a higher respiration rates. These data illustrate the importance of maintaining cold-chain conditions for wild rocket to preserve key glucosinolate compounds and prevent sulfide formation via bacterial propagation and anaerobic respiration.

1. Introduction

Rocket species (*Eruca sativa* and *Diplotaxis tenuifolia*) are Ready-To-Eat (RTE) salad leaves that are rapidly gaining popularity. RTE rocket is pre-processed material that is washed and bagged, ready for human consumption. Most RTE products are considered nutritious and convenient ways to increase dietary intake of fruits and vegetables (Cavaiuolo et al., 2015). These are, however, prepared without heat treatments, which is not necessary or required for human consumption. RTE salads have a neutral pH, are abundant in sugars, amino acids, and other phytochemicals that can be metabolized by bacteria. This means the foods can become ideal media for microbial growth if not handled correctly (Rico et al., 2007). Salad processors apply a variety of methods to reduce and prevent microbial proliferation, such as the addition of chlorine to wash water (< 50 ppm), multiple rinsing and drying steps, and modified atmosphere. Not all of these methods are universally applied or required by law, and their effectiveness at preventing microbial increases has not been rigorously or scientifically tested.

Processing of fresh RTE salads involves many steps, including those that may cause plant tissues to wound and collapse. This can include the transfer of leaves in bulk from crates and into wash lines, the impact of water flow and plunge pools to dislodge soil and detritus, as well as centrifugal force exerted when leaves are spin-dried. Injury inflicted during the preparation of produce promotes physiological changes that hasten loss of visual product quality, leading to the removal of the protective epidermal layer and exposure of internal cells (Brecht, 1995); these changes provide easy access for microbes. The combination of wounding and an increased microbial population alters physiological processes within the tissue of RTE products such as rocket. This increases respiration, and also affects the synthesis and accumulation of certain phytochemicals that eventually change the nutritive

* Corresponding author.
E-mail address: luke.bell@reading.ac.uk (L. Bell).

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profile of leaves (Barth et al., 2009). All these factors will ultimately affect physical appearance and the concentrations of beneficial phytochemicals of RTE rocket.

The microbiological quality and safety of RTE produce have become major concerns, to both service providers and consumers. The US Food and Drug Administration Food Code has recommended that packaged fresh-cut leafy green vegetables (such as rocket) be kept at 5 °C or below during transport, storage and retail display (Kou et al., 2015). However, in 'real' supply chain situations, a continuous cold-chain is difficult to achieve (Koseki and Isobe, 2005; Kou et al., 2015). Higher temperatures may be experienced during transportation and handling from the factory to the retailer, during display at the retailer's premises, and during transport home after purchase by the consumer. Once bought, the consumer may not eat the entire product in one meal, and may keep it in their home refrigerator for later consumption. All these practices have implications for the microbial loading, freshness and nutritive values of the products.

The effect of temperature on microbial load, quality, and generation of flavour compounds of fresh RTE salads are well studied; however, most experiments are conducted under constant temperature regimes. In contrast, this paper will determine the effects of different and fluctuating temperature regimes that may occur along the 'real world' post processing supply chain of fresh RTE rocket, and assess the impacts these have on microbial loads and production of volatile organic compounds (VOCs).

Higher ambient temperature would normally enhance multiplication of mesophile and psychrotroph bacteria within salad bags, thus increasing the abundance present on the leaves, especially when storage is prolonged (Kou et al., 2015). As the growth of bacteria is a function of time and temperature of storage, we hypothesised that microbial loads would be significantly elevated with increasing storage time and duration of exposure to temperatures > 4 °C. We additionally hypothesised that short periods of abuse temperature would not increase bacterial numbers significantly, providing cool-chain temperatures were maintained thereafter. This would therefore be of practical knowledge to processors and supermarkets, where short-term breaks are sometimes necessary for the transfer and storage of material at suboptimal temperature conditions.

Bags of rocket material are sometimes opened by the consumer in the home, where only portions of leaves are consumed; the rest are left for later consumption. Leaves that are disturbed by unwashed hands in this way could potentially cause cross-contamination, and therefore we also theorized that leaves would contain a higher bacterial load in this scenario than in bags of the same relative shelf life stage that had not been opened. The determination of this effect will allow consumers to better judge the impact of only taking small portions of salad versus consuming leaves within bags at the same time.

Due to the role of temperature in affecting bacterial growth, we hypothesised that summer-grown rocket leaves would contain relatively higher loads compared to those produced in winter. Winter-grown rocket by contrast was expected to have a lower respiration rate, which would negatively affect bacterial propagation.

A special emphasis was given to evaluate the effects of temperature and bagging treatments on glucosinolates (GSLs) in our experiment due to their role in determining taste of the rocket (Pasini et al., 2011), the anticancer properties of breakdown isothiocyanate (ITC) hydrolysis products (Hayes et al., 2008; Ombra et al., 2017), and purported antimicrobial properties (Khoobchandani et al., 2010; Koubaa et al., 2015). We postulated that variations in bacterial loads would affect GSL concentrations, which are important for nutritional qualities of the crop, as well as the abundance of volatile compounds within the bag headspace.

2. Materials and methods

2.1. Plant materials & experimental treatments

All plant materials were grown and harvested according to standard commercial practices, and obtained directly from rocket salad processors in the UK. Plants were harvested at a commercially relevant growth stage (32–38 days of growth). Samples were harvested in Italy and shipped by lorry to the UK under cool-chain conditions; the duration lasted approximately three days. Winter produce originated from the Campania region (mean average daily temperature high was 11 °C, and the average low was 8 °C; average precipitation was 13.9 mm, and average humidity was 73%), and summer produce from the Veneto region (mean average daily temperature high was 31 °C, and the average low was 26 °C; precipitation was 0 mm, and average humidity was 61%). For reasons of commercial sensitivity specific farm locations within these areas will not be given. Bags of rocket from each specified shipment were sampled independently for each analysis. One bag is equivalent to one experimental replicate in each respective analysis; no two analyses were sampled from the same bag. Three replicates were used per treatment, per analysis unless otherwise stated.

The sampling scheme followed for chemical, microbial and atmospheric analyses are presented in Fig. 1, with additional explanation provided in Table S1. Briefly, five sampling scenarios were tested based upon differing hypothetical temperature change scenarios. These were:

1 Temperature abuse by a hypothetical supplier with an initial period of 25 °C temperature abuse and 13 °C storage for one day (TAS4H), five days (TASDUD), and an additional two days at 4 °C (TASDUD + 2).
2 Temperature abuse by a hypothetical consumer with an initial period of 25 °C temperature abuse and return to 4 °C storage for one day (TAC4H), five days (TACDUD), and seven days (TACDUD + 2).
3 Temperature abuse by a hypothetical supplier at 13 °C storage for five days (NPDUD), and an additional two days at 4 °C (NPDUD + 2).
4 Cool chain maintenance at 4 °C for five (EPCDUD) and seven days (EPCDUD + 2) in closed bags.
5 Cool chain maintenance at 4 °C for five (EPODUD) and seven days (EPODUD + 2) in open bags.

2.1.1. Volatile organic chemical & glucosinolate experiment

The rocket cultivar used was *Reset* (*D. tenuifolia*), and was a second cut crop grown in southern Italy (Campania region). Samples of washed wild rocket used in this experiment were processed at a facility in the UK (which will not be named for reasons of commercial sensitivity), and transported on the day of intake to the Department of Food and Nutritional Sciences, University of Reading; journey duration was approximately two hours. The cold chain was maintained at < 5 °C during transportation from Italy to UK, as well as from the supplier to the University of Reading in a temperature-controlled lorry. More details on the industrial processing of the leaves used in this experiment are as described in Bell et al. (2017). The materials were then subjected to different handling and storage conditions upon arrival, in triplicate. The treatments used are summarized in Fig. 1 and Table S1. The temperatures selected for experimentation are based upon and Bell et al. (2017). Each experimental unit was comprised of one bag (70 g) with unmodified atmosphere. The timeline, temperature changes, and points of sampling are presented in Fig. 1.

2.1.2. Microbial count & gas composition seasonal experiment

Bagged plant materials in unmodified atmosphere were obtained as in 2.1.1. For winter grown leaves (from the Campania region), the crop was produced from January to February using wild rocket cultivar *Reset* (second cut) with 38 days growth cycle after sowing. The summer crop was produced from July to August (in the Veneto region), using cultivar

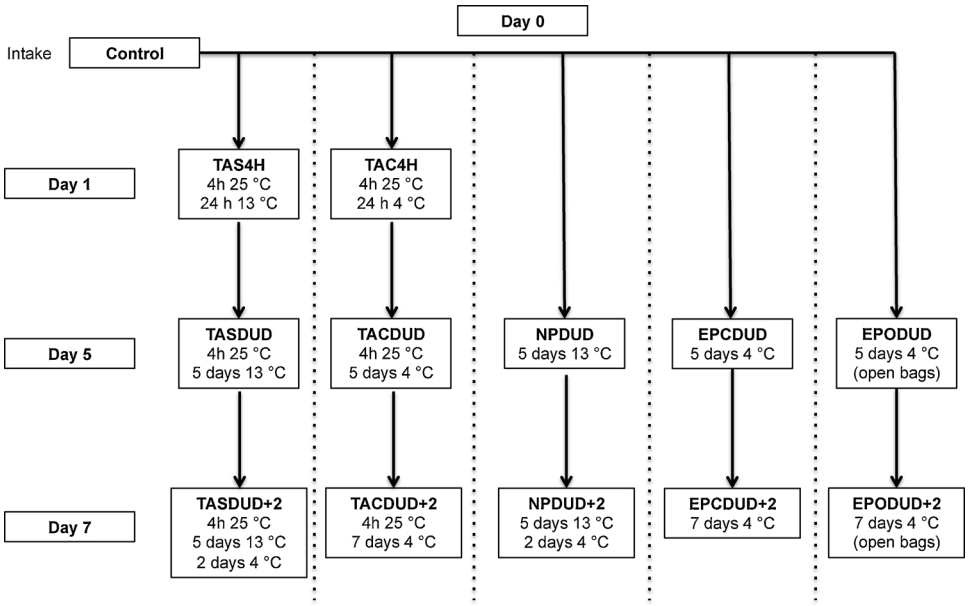

Fig. 1. Timeline of sampling points for determination of microbial loads, volatile organic compounds, atmospheric composition, and glucosinolate concentrations of wild rocket under differing temperature and bag opening scenarios that represent different supply chain and consumer behaviors. Abbreviations: TAS4H, temperature abuse by a hypothetical supplier on day 1 (temperature abuse defined as four hours at 25 °C) followed by subsequent maintenance at 13 °C for a further 24 h before sampling to mimic conditions within a retail store*; TASDUD, temperature abuse and then subsequent maintenance at 13 °C until the display until date; TASDUD + 2, temperature abuse and then maintenance at 13 °C until two days after the display until date; TAC4H, temperature abuse by a hypothetical consumer for four hours post-purchase followed by subsequent maintenance at 4 °C for a further 24 h before sampling to mimic a domestic refrigerator; TACDUD, temperature abuse and then subsequent maintenance at 4 °C until the display until date; TACDUD + 2, temperature abuse and then maintenance at 4 °C until two days after the display until date; NPDUD, not "purchased" until display until date so maintained at retail temperature 13 °C for five days; NPDUD + 2, not "purchased" until display until date and subsequently maintained at 4 °C to mimic a domestic refrigerator for a further two days; EPCDUD, early "purchase" by consumer so the pack was immediately transferred to 4 °C and then kept closed until the display until date; EPCDUD + 2, early "purchase" by consumer so the pack was immediately transferred to 4 °C and then kept closed until two days after the display until date; EPODUD, early "purchase" by consumer who then opened the bag to remove a portion and retained the rest of the contents at 4 °C until the display until date; EPODUD + 2, early "purchase" by consumer who then opened the bag to remove a portion and retained the rest of the contents at 4 °C until two days after the display until date. See Table S1 for full descriptions. * Retail store temperature was set at 13 °C for this experiment. Whilst many store shelves manage to achieve lower temperatures than this the temperature used is representative of those recorded in stores near the front of the chill cabinet when open shelves are used, as is common in UK retailers.

Extrema (second cut), with 32 days growth cycle after sowing. The changing of variety according to the season is common industrial practice. The post processing shelf life treatments used in these experiments were as described in Fig. 1.

2.2. Microbial count analyses

2.2.1. Preparation of nutrient agar for total plate count

11.75 g of standard plate count agar (Oxoid Ltd, Basingstoke, Hampshire, UK) was diluted in 500 mL of distilled water in a 500 mL bottle, and stirred on a hot plate using a magnetic stirrer until boiling, giving a final concentration of 2.4% (w/v). Agar was subsequently sterilized for 15 min at 121 °C. The media were kept at 45 °C in a water bath to maintain the liquid state. The media were transferred to Eppendorf tubes prior to usage, and temperature was taken using sterile thermometer to confirm the temperature did not exceed 45 °C.

2.2.2. Preparation of maximum recovery diluent for sample preparation & enumeration

9.5 g of maximum recovery diluent (MRD; Sigma Aldrich, Missouri, USA) was diluted in 1 L of distilled water (0.95%; w/v) in 1 L bottle and stirred using magnetic stirrer until completely dissolved. MRD diluents were poured into 100 mL bottles, with 90 mL in each bottle for sample preparation, and 9 mL for enumeration, and sterilized for 15 min at 121 °C. The mixture was cooled in a laminar flow hood before being used, or was kept in a 4 °C cold room for longer-term storage.

2.2.3. Total plate count

10 g of leaves from one bag, per replicate, per treatment, were added to 90 mL of MRD in a stomacher bag and shaken for 120 s, creating a 10^{-1} dilution (w/v). 0.1 mL of the homogenized/inoculum was sampled from the bag and serially diluted to obtain 10^{-2}, 10^{-3}, 10^{-4} until 10^{-7}. The homogenate (1 mL) was sampled from the bag and it was serially diluted to obtain 10^{-1}, 10^{-2} and 10^{-3} dilution. 1 mL of the respective solutions were placed on the nutrient agar plates using the pour plate technique (< 45 °C) and the plates were swirled to mix evenly. Inoculated plates were allowed to cool at room temperature until the liquid solidified. Plates then were incubated at 30 °C in inverted condition. After 72 ± 3 h of incubation the number of colonies per plate were counted using a colony counter. Plates with colonies > 300 were labeled with TNTC (too numerous to count); plates with colonies < 30 were discarded.

2.2.4. Scanning electron microscopy

SEM was performed using Quanta 600 Environmental Scanning Electron microscope (FEI, Hillsboro, OR, USA) at the Centre for Advanced Microscopy, University of Reading, UK. Two methods of sample preparation were employed which were: the chemical fixation method (Conventional SEM) and cryo-method (Cryo-SEM).

Leaves from independent bags were prepared and fixed immediately upon arrival at the Department of Food and Nutritional Sciences, University of Reading. Samples were taken at the time points Day 0 and Day 6 of storage at the temperatures used in preliminary experiment B. Samples were cut into small squares (3 mm x 3 mm) to facilitate penetration and were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 25 mM HEPES buffer. The samples were kept in the fixative overnight at 5 °C. Samples were washed in HEPES buffer for 15 min and were dehydrated through a serial dilution of acetone starting with 30%, 50%, 70%, 80%, 90%, 100%, and twice more with 100% dry solvent with molecular sieve, with a 15 min interval for every dilution. Samples were critical point-dried using CO_2 and sputter coated with gold for 2.5 min before the images were viewed in a Quanta 6000 Environmental SEM, operated at 20 kV.

For the Cryo-SEM method leaf pieces were cut into small squares (3 mm × 3 mm), mounted and fixed on a sample shuttle using colloidal graphite and OCT low temperature adhesive. Samples were plunge-frozen in slushed liquid nitrogen at -210 °C until boiling terminated, before being transferred into the chamber under vacuum. Each sample

was placed into the cryo-preparation chamber attached to the Quanta 600 Environmental Scanning Electron microscope. The temperature of the chamber was then raised to −90 °C for 10 min to sublimate any condensed ice from the surface gained during sample transfer. The temperature of the sample was then reduced to -135 °C. To avoid sample charging problems while searching for a suitable site, the sample was sputter coated with thin layer of platinum for 30 s. Afterwards, samples were loaded on the cryo-stage, which was maintained at -135 °C using nitrogen liquid. Imaging was performed using an acceleration voltage of 10 kV.

2.3. Volatile organic compound analysis

2.3.1. Solid phase microextraction for volatile organic compounds

Independent bagged rocket salad samples for each treatment (70 g) were analysed by SPME and were left at room temperature for ten minutes prior to sampling. An SPME device (Supelco, Bellefonte, PA, USA), containing a 1 cm Stable-flex fiber coated with 50/30 µm DVB/Carboxen on PDMS was used. The fiber was conditioned before use by heating it in a gas chromatograph injection port at 250 °C for 30 min.

A steel needle was used to make a hole in the top of each sample bag, through which the SPME syringe needle was inserted. The fiber was exposed to the headspace above the sample for 30 min (Taviera et al., 2009). The bag and syringe were supported with clamps so that the fiber did not touch the rocket leaves or the bag. After extraction the SPME device was removed from the sample bag and inserted into the injection port of the GC–MS system. 33 bags of rocket were sampled in total.

2.3.2. Gas chromatography mass spectrometry

All analyses were performed on a Hewlett-Packard 5972 mass spectrometer (Palo Alto, CA, USA), coupled to a 5890 Series II gas chromatograph with G1034C ChemStation software. The VOCs on each SPME fiber were desorbed for 3 min in a split/splitless injection port, held at 250 °C on to the front of a Stabilwax DA fused silica capillary column (30 m, 0.25 mm i.d., 0.50 µm film thickness; Restek Corporation, Bellefonte, PA, USA). The front of the column was shaped into five small loops in a coil, which were cooled in dry ice and contained within a 250 mL beaker. The injection port was set in splitless mode, with the splitter opening after 3 min. During desorption the oven was set to 40 °C. After desorption, the beaker containing the dry ice was immediately removed. The oven was maintained at 40 °C for a further 2 min and the temperature was raised at 5 °C per min to 250 °C and hold for 5 min. Helium at 12.7 psi was used as the carrier gas, resulting in a flow of 1.5 mL min^{-1} at 40 °C.

The mass spectrometer was operated in electron impact mode with electron energy of 70 eV, and an emission current of 50 µA. The ion source was maintained at 170 °C. The mass spectrometer scanned from *m/z* 29 to *m/z* 450 at 1.81 scans.s^{-1}. Volatile compounds were identified by comparison of each mass spectrum with spectra from authentic compounds analysed in our laboratory, or from the NIST mass spectral database (NIST/EPA/NIH Mass Spectral database, 2011), or spectra published elsewhere. To confirm the identification, the linear retention index (LRI) was calculated for each volatile compound using the retention times of a homologous series of C6–C25 n-alkanes (Sigma) and by comparing the LRI with those of authentic reference standards analysed under similar conditions. Alkane standard was exposed to an SPME fiber for an identical duration as each sample, and run before and after each batch of samples every day. Relative abundances of each compound collected from the headspace of the samples were compared by measuring their peak areas obtained by integration using the ChemStation integrator.

2.4. Glucosinolate analysis by LC–MS

GSL extraction and LC–MS analysis were conducted on freeze dried material according to the protocol by Bell et al. (2015) using identical consumables and equipment.

2.5. Analysis of oxygen, carbon dioxide

An oxygen and carbon dioxide gas analyzer for modified atmospheric packaging (MAP; Oxybaby, Witt, Germany) was used to measure content (%) in independent rocket packages ($n = 3$) delivered from the processing facility.

2.6. Statistical analysis

Data on total plate counts were subjected to analysis of variance (ANOVA) using Statistical Analysis System (ver. 9.3; SAS Institute, Cary, NC, USA). Mean differences between treatments were compared using Duncan's Multiple Range Test (DMRT; $P < 0.05$). Graphs were then plotted using Excel spread sheet.

Protected analysis of variance (ANOVA) was carried out on the quantitative data for each analysis (VOC, gas composition, & GSLs; XLStat, Addinsoft, Paris, France). For those compounds exhibiting significant difference in the ANOVA, DMRT was applied to determine which pairwise comparisons differed significantly ($P < 0.05$). Data collected for VOC and GSL analyses were separately used in Principal Component Analysis (PCA, Pearson *n*-1; XLStat) where relationships were determined by coefficient analysis.

3. Results

3.1. Preliminary experiments

Preliminary experiments using samples kept at 4 °C, 13 °C or 20 °C for six days, with either open or closed bags, demonstrated that the bacterial numbers increased significantly in line with storage temperature (Table S1, Fig. S1). A 4 °C temperature was sufficient to maintain bacterial growth at minimal levels whether or not the bag was opened; at higher temperatures the open bag gave rise to significantly higher bacterial counts than from a closed bag system (Figs. S2 and S3). These observations gave rise to the design of the experiments reported hereafter, which use rocket of known provenance to test the hypothesis that temperature abuse during the supply chain gives rise to an increased risk of bacterial growth and results in detrimental impacts on multiple quality parameters.

3.2. Growth of bacteria in bagged fresh RTE wild rocket during various shelf life scenarios

Bacterial abundance (measured as Total Plate Counts – TPC) in RTE rocket varied along the various points of the post processing shelf life (Fig. 2), indicating that the increase in TPC depended on the temperature, handling and storage conditions. Rocket kept under constant 13 °C conditions (until the DUD; TASDUD) contained the highest TPC (10.2-log cfu.g^{-1}). This was significantly higher than all other samples. In turn, other samples at this temperature (but varying in their sampling date: NPDUD, NPDUD + 2, TASDUD + 2), with their respective TPCs of 10.0-log cfu.g^{-1}, were significantly higher than the other remaining time points. Rocket leaves subjected to other treatments, such as abuse temperature with subsequent storage at 4 °C (TAC4H, TACDUD, TACDUD + 2) and those with no abuse temperature (EPCDUD, EPODUD, EPCDUD + 2, EPODUD + 2) were not significantly different from the control.

The results show the importance of temperature in determining the growth of bacteria in RTE rocket salads. The high TPC in TASDUD was linked to the prolonged exposure to high temperatures (25 and 13 °C). Surprisingly, the TPC of treatment TASDUD was significantly higher than those in treatment TASDUD + 2 by 0.2-log cfu.g^{-1}, even though the latter had been subjected to similar conditions plus two extra days

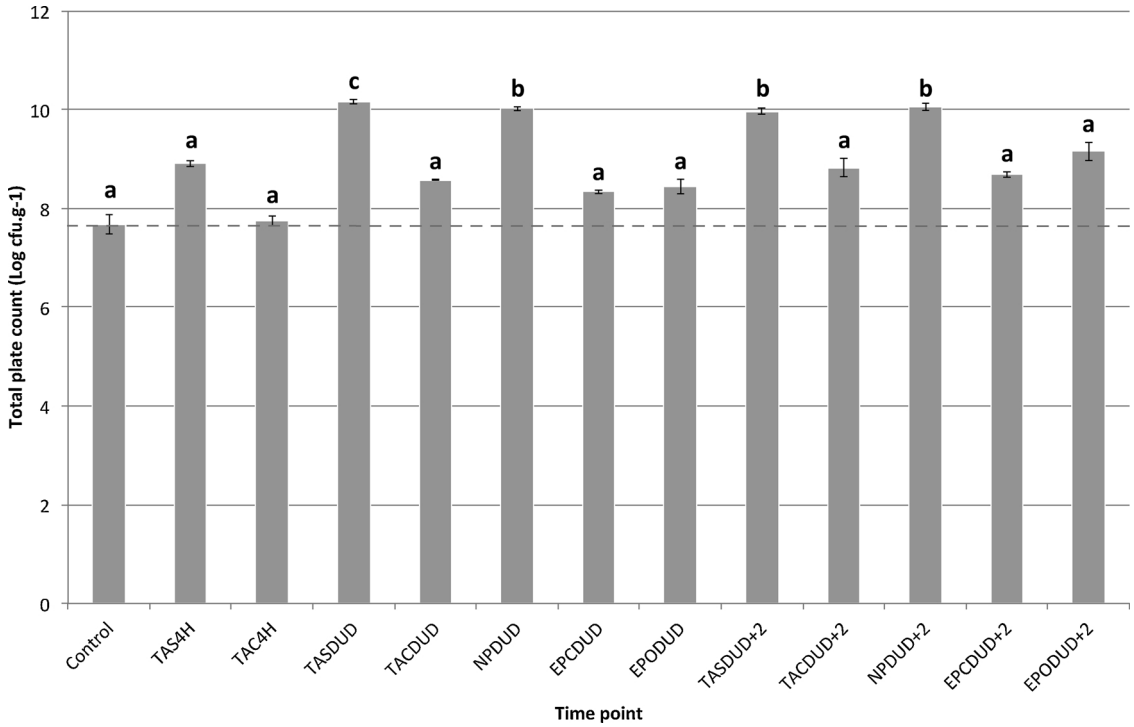

Fig. 2. Total plate counts of bacteria found in ready-to-eat wild rocket salad bags subjected to different handling and storage conditions. Letters above bars indicate significant differences according to Duncan Multiple Range Test ($P < 0.05$). Dotted line indicates TPC at control. Error bars refer to the standard error of the mean based upon three experimental replicates. Refer to Fig. 1 and Table S1 for treatment definitions.

in storage at 4 °C. This could be the result of microbial death due to insufficient nutritional supply or unfavorable atmospheric conditions at the end of shelf life. Regardless, both samples exposed to high temperature for 4 h, followed by storage at 13 °C, produced TPCs significantly greater than the control and other samples that were not subject to temperature abuse.

TPC did not significantly increase when rocket was kept at 4 °C, although the samples had been previously exposed to 25 °C for 4 h (Fig. 2). This supports our hypothesis that short periods of abuse temperature do not adversely affect produce, provided that it is returned to cool-chain conditions. Similar results were shown in rocket stored at 4 °C for both opened and closed bags (EPCDUD and EPODUD). Storing rocket at 4 °C for seven days, either in closed or opened bags until DUD (EPCDUD + 2 and EPODUD + 2), similarly did not significantly increase the growth of bacteria, and the TPC of the leaves were not significantly different from the control. Conversely, rocket that was kept under display temperature (13 °C) showed a high TPC on the DUD, even though not exposed to abuse temperature or hand contamination.

3.3. Changes in the relative abundance of volatile organic compounds & glucosinolates of fresh RTE wild rocket during various shelf life scenarios

3.3.1. Volatile organic compounds

VOCs emitted from the rocket leaves during shelf life were significantly affected by storage conditions, as shown in Table 1. Volatiles from seven different groups of compounds were detected: sulfides, alcohols, ketones, furans, aldehydes, esters, and alkanes; as well as three unknown compounds.

3.3.1.1. Sulfides. Four sulfide compounds were detected in the samples: dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and dimethyl sulfoxide (DMSO). DMS was not detected in the first four treatments; fresh leaves (control), TAC4H, TAS4H, and EPCDUD. The results show that the abundance of DMS in TACDUD, TASDUD, EPCDUD + 2, TACDUD + 2 are not significantly different in terms of relative abundance. The compound was highest in leaves that were purchased and sampled on the DUD (NPDUD), abusive temperature by the supplier and sampled two days after the DUD (TASDUD + 2), and those that were purchased and sampled two days after the DUD (NPDUD + 2).

Except for the control (Day 0), DMDS was detected in all other treatments. Leaves that experienced abuse temperature (25 °C) for 4 h before being transferred to 4 °C for 24 h (TAC4H) contained the lowest abundance of DMDS, but the value did not differ significantly from TAS24, TASDUD, TASDUD + 2, NPDUD + 2. Leaves of EPCDUD generated less compared to those that were in the EPCDUD + 2 treatment. Amongst treatments, leaves that were sampled two days after display until date (EPCDUD + 2) generated the greatest abundance of DMDS. Comparatively, bags that were temperature abused for 4 h and sampled on the DUD (TASDUD), temperature abused for 4 h and sampled on two days after the DUD (TASDUD + 2), and leaves that were 'purchased' and sampled two days after the DUD (NPDUD + 2), produced lower abundance of DMDS than EPCDUD and EPCDUD + 2. These data suggest that short-term temperature abuse (at 25 °C) does not significantly affect the generation of DMDS.

DMTS was only detected in the headspace of two treatments: TASDUD and TASDUD + 2, which suggests that long periods of inappropriate supermarket temperature storage (13 °C) influences the abundance of this compound. As with the other sulfides, the occurrence of DMSO differed significantly, whereby rocket in treatment NPDUD generated the highest relative amount. Leaves that were stored at high temperature (13 °C) generated significantly higher abundances of DMSO compared to those kept continuously at 4 °C (EPCDUD). The amount of the compound produced by rocket at Day 0, TAC4H, TAS4H, EPCDUD, TACDUD, and TACDUD + 2 were not significantly different. DMSO appears to be the least sensitive to changes in temperature and duration of storage, with the exception of NPDUD and NPDUD + 2. The DMSO abundance tended to increase after long duration of storage, but the values were not significantly different.

Table 1
Relative abundance[a] of volatile organic compounds identified within the bag headspace of fresh ready-to-eat wild rocket at different points in the post processing supply chain. See Table 1 for descriptions of each scenario.

Compound class	Compound	LRI[b]	ID[c]	Control (Day 0)	TAC4H	TAS4H	EPCDUD	TACDUD	TASDUD	NPDUD	EPCDUD + 2	TACDUD + 2	TASDUD + 2	NPDUD + 2
Sulfide	Dimethyl sulfide	723	B	nd	nd	nd	nd	487 bc	458 bc	582 ab	494 bc	348 c	726 a	721 a
	Dimethyl disulfide	1074	A	nd	361 f	1004 ef	9942 b	8505 bc	2854 def	6139 bcd	19259 a	8505 bc	5471 bcde	4882 cdef
	Dimethyl trisulfide	1383	A	nd	nd	nd	nd	nd	952 a	nd	nd	nd	1032 a	nd
	Dimethyl sulfoxide	1575	A	418 cd	668 cd	248 d	580 cd	685 cd	1676 bc	6747 a	1065 cd	581 cd	1486 bcd	270 b
Alcohol	1-butanol	1141	A	nd	nd	nd	nd	nd	nd	1046 a	nd	nd	nd	470 b
	1-penten-3-ol	1167	A	nd	nd	nd	nd	nd	1610 a	1697 a	nd	nd	2403 a	1821 a
	(*Z*)-2-penten-1-ol	1325	A	nd	nd	nd	nd	nd	603 b	569 b	nd	nd	1893 a	210 bc
	1-hexanol	1356	A	nd	nd	nd	nd	nd	192 bc	257 b	nd	nd	1912 a	214 bc
	(*Z*)-3-hexen-1-ol	1387	A	nd	nd	nd	nd	nd	283 c	1107 b	nd	nd	6746 a	1286 b
	Phenethyl alcohol	1917	A	nd	nd	nd	nd	nd	205 c	956 ab	nd	nd	1238 a	457 bc
Ketone	3-pentanone	977	A	nd	nd	nd	nd	nd	4906 a	4947 a	nd	nd	7489 a	5156 a
	3-methyl-2-butanone	983	B	nd	nd	nd	nd	nd	1469 ab	926 b	nd	nd	1197 ab	1838 a
	Dihydro-2(3 H)-thiophenone	1650	B	nd	nd	nd	nd	74 b	265 ab	414 a	201 ab	396 a	300 ab	391 a
	Acetoin	1293	A	nd	nd	nd	nd	nd	nd	nd	nd	nd	2386 a	nd
Furan	2-methylfuran	886	B	488 d	1265 dc	989 d	1656 dc	1407 dc	4182 bc	6316 ab	190 cd	182 d	7504 a	7242 a
	3-ethylfuran	951	B	nd	nd	nd	387 bc	nd	1552 a	1465 a	nd	nd	1096 ab	1150 ab
Aldehyde	Benzaldehyde	1529	A	nd	nd	nd	nd	nd	3017 a	3680 a	nd	243 a	4374 a	3762 a
	2-thiophenecarboxaldehyde	1703	A	nd	nd	nd	nd	nd	218 c	652 a	nd	nd	465 b	222 c
Ester	(*Z*)-3-hexenyl-3-methylbutanoate	1490	A	nd	nd	nd	nd	nd	711 bc	5181 a	nd	nd	1529 b	1812 b
Alkane	2,4-dithiapentane	1289	A	nd	nd	nd	263 de	242 de	504 cd	1136 a	193 de	127 e	710 bc	893 ab
Unknown	< Unknown 1 >	1465		nd	465 bc	266 bc	366 bc	365 bc	593 bc	2684 a	nd	308 bc	1097 b	1191 b
	< Unknown 2 >	2013		169 b	261 b	190 b	410 b	293 b	213 b	670 ab	147 b	192 b	4795 a	1096 a
	< Unknown 3 >	2462		nd	nd	nd	nd	nd	nd	nd	211 b	424 b	1354 a	143 b

[a] Values are peak area means of three replicates divided by 10^2; values within rows with the same letters do not differ significantly based on Duncan Multiple Range Test at $P < 0.05$; nd = not detected.
[b] Linear retention index on a Stabilwax DA column.
[c] A, mass spectrum and LRI agree with those of authentic compound; B, mass spectrum agrees with reference spectrum in the NIST/EPA/NIH mass spectra database and LRI agree with those in literature.

3.3.1.2. Alcohols. Six alcohols were detected in rocket samples: 1-butanol, 1-penten-3-ol, (*Z*)-2-penten-1-ol, 1-hexanol, (*Z*)-3-hexen-1-ol, and phenethyl alcohol. The generation patterns of these compounds were similar, with the exception of 1-butanol. Alcohols were only detected in leaves that were exposed to long storage durations and at high temperature. Overall, rocket leaves generated high amounts of alcohols under the TASDUD + 2 treatment, and also for treatments NPDUD and NPDUD + 2. No alcohols were detected at Day 0, or in those that were subjected to treatments TAS4H, TAC24, EPCDUD, EPCDUD + 2 and TACDUD + 2. The results clearly demonstrate that short-term abuse temperature at 25 °C for 4 h, and exposure to 13 °C thereafter, aggravated alcohol production in rocket leaves.

The abundance of 1-penten-3-ol in samples, with or without temperature abuse, did not significantly differ between each sample; however, this compound was affected by the duration of storage. At the earlier days of storage (observed in control, TAC4H and TAS4H), 1-penten-3-ol was not detected, but later it was found at TASDUD and TASDUD + 2, which were at five and seven days of shelf life storage, respectively.

In contrast to 1-penten-3-ol, (*Z*)-2-penten-1-ol abundance was influenced by the temperature abuse treatment, but the significant difference was only seen in leaves stored up to two days after the expiry date (TASDUD + 2 and NPDUD + 2). This alcohol was also affected by the duration of storage, particularly in leaves that were kept at high temperature. It was not detected in TAS4H, but it was found in TASDUD, and then increased markedly in TASDUD + 2 leaves. The microbial TPC counts were also highest under TASDUD, TASDUD + 2, and NPDUD (Fig. 2), supporting the hypothesis on the possible positive relationship between the number of bacteria and the generation of certain species of VOCs in fresh RTE rocket.

3.3.1.3. Ketones. The generation of ketones among rocket samples differed markedly by treatment, and the pattern of response varied with each compound. The generation of 3-pentanone and 3-methyl-2-butanone was most similar in leaves that were subjected to treatments TASDUD, NPDUD, TASDUD + 2 and NPDUD + 2. The relative amount of 3-pentanone released was much higher compared to 3-methyl-2-butanone; these were detected in leaves stored at 13 °C, but temperature abuse had no significant effect. Duration of storage affected the abundance of the compounds but only until the DUD; extending storage two days after DUD had no significant effect.

In contrast, dihydro-2(3 H)-thiophenone was detected more frequently, but it appeared in smaller relative amounts. It was also detected in leaves kept at low temperature, but only in those that experienced temperature abuse (TACDUD and TACDUD + 2). The results suggest that exposure to 25 °C for 4 h produced a significant effect on the abundance of dihydro-2(3H-thiophenone in rocket leaves.

3.3.1.4. Furans. Two furan compounds were detected: 2-methylfuran and 3-ethylfuran. Between the two, 2-methylfuran was found in leaves of all treatments, while 3-ethylfuran was only detected in five. Among these, leaves subjected to treatments NPDUD, TASDUD + 2, and NPDUD + 2 produced the highest relative amounts of 2-methylfuran. Generation was affected by temperature and storage duration, whereby the compound increased significantly. 3-Ethylfuran was detected in rocket samples that were kept mainly at high temperature.

3.3.1.5. Aldehydes. Between the two aldehydes detected, benzaldehyde was found to be more abundant compared to 2-thiophenecarboxaldehyde. The occurrence of these two compounds was similar and detected in leaves that were temperature abused at 25 °C for 4 h, stored at 13 °C, and sampled two days after the display until date (TACDUD + 2). Among the treatments in which benzaldehyde was detected, the difference was not significant. In contrast, among the leaves of treatments that contained 2-thiophenecarboxaldehyde, the abundance differed significantly. Leaves that were purchased and sampled on the DUD (NPDUD) contained the highest abundance, and this was followed by TASDUD + 2, NPDUD + 2, and TASDUD. Exposing rocket to high temperature significantly affected the abundance of both compounds. Keeping the leaves for a longer duration of storage at 13 °C also influenced the presence of benzaldehyde and 2-thiophencarboxaldehyde.

3.3.1.6. Esters. The only ester detected was (*Z*)-3-hexenyl-3-methylbutanoate, in four out of the 11 treatments (TASDUD, NPDUD, TASDUD + 2 and in NPDUD + 2). Leaves that were purchased and sampled on the display until date (NPDUD) generated the highest amount, followed by NPDUD + 2, TASDUD + 2, and TASDUD. The difference between the abundance of the compound from NPDUD + 2 and TASDUD + 2 was not significant. (*Z*)-3-hexenyl-3-methylbutanoate was positively affected by both storage duration and temperature, as the compound was only detected in leaves that were kept at high temperature and until after the DUD.

3.3.1.7. Alkanes. The alkane 2,4-dithiapentane was not detected in control rocket leaves (Day 0), TAC4H, or TAS4H. Like the furans, aldehydes and ester compounds, the highest amount of 2,4-dithiapentane was detected in leaves that were subjected to NPDUD, and this was similar to those treated under NPDUD + 2, but was not significantly different. 2,4-dithiapentane was high in leaves that were kept at high temperature, regardless of exposure duration, but only at the point of the DUD and later.

3.3.2. Glucosinolates

Five GSL compounds were detected in the bagged fresh RTE wild rocket used in this experiment (Table 2), and the concentrations of all varied significantly between treatments. The GSLs identified were glucosativin, glucoerucin, glucoraphanin, diglucothiobeinin and dimeric glucosativin (dimeric 4-mercaptobutyl; DMB).

The treatments that experienced abuse temperature but were sampled after 24 h (TAS4H and TAC4H), and leaves stored at low temperature (4 °C; EPCDUD, EPODUD, TACDUD, and TACDUD + 2) contained the highest concentrations of glucosativin, and the mean values of this compound did not differ significantly for these treatments. The average glucosativin content in these leaves was 4.9 $mg.g^{-1}$ dw. Glucosativin was not detected at EPODUD + 2, indicating that it may have been completely converted to DMB (7.1 $mg.g^{-1}$ dw), though the exact biological mechanism by which this may occur is unknown. Glucosativin is responsible for the generation of sativin, which creates the characteristic aroma and flavour of rocket leaves (Raffo et al., 2018), and it is therefore important to maintain throughout the supply chain. Our results show that the concentration of glucosativin seems to be lower when stored at 13 °C compared to those stored at 4 °C. The negative effects of high temperature on leaves are evident in TASDUD, NPDUD, TASDUD + 2, and NPDUD + 2 treatments. Diglucothiobeinin was not detected in leaves of TASDUD, TACDUD, TACDUD + 2, EPCDUD, and EPCDUD + 2. Leaves in treatments TAS4H and EPODUD contained significantly higher concentrations of this compound than any of the others (both 3.6 $mg.g^{-1}$ dw).

TASDUD + 2 and TAC4H contained the highest concentrations of glucoerucin (4.9 and 4.5 $mg.g^{-1}$ dw, respectively). By contrast, the compound was not detected in TAS4H, TACDUD, TACDUD + 2, NPDUD, NPDUD + 2, or EPODUD. This suggests that high storage temperature is also detrimental to the occurrence of this compound. The concentration of glucoraphanin was highest in EPCDUD + 2 samples (4.3 $mg.g^{-1}$ dw), and this was significantly higher than those recorded for all other treatments. Under this condition cold chain conditions were maintained, which suggests that prolonged storage without temperature abuse enhances glucoraphanin concentrations, rather than reduces it. It is therefore apparent that maintaining cool-chain temperatures is likely to preserve health-related GSLs, as well as

Table 2
Concentrations of glucosinolates in fresh ready-to-eat wild rocket at different points of post processing shelf life.

Treatments	Glucosinolate concentration (mg. g^{-1} dw, sinigrin hydrate equivalent)					
	Glucoraphanin	Diglucothiobeinin	Glucosativin	Glucoerucin	Dimeric glucosativin	Total glucosinolates
Control	2.35 c	0.65 c	9.50 a	1.81 c	8.48 a	22.79 ab
TAS4H	nd	3.56 a	6.34 a	nd	nd	9.90 de
TASDUD	3.48 b	nd	6.15 a	1.81 c	1.22 cd	12.66 c
TASDUD + 2	3.36 bc	2.32 bc	4.67 abc	4.91 a	1.64 c	20.82 b
TAC4H	3.47 b	1.67 c	6.05 ab	4.54 a	4.85 b	24.17 a
TACDUD	3.53 b	nd	6.03 ab	nd	nd	9.56 de
TACDUD + 2	3.13 bc	nd	2.89 def	nd	1.58 c	7.60 e
NPDUD	3.08 bc	3.16 ab	1.92 f	nd	6.52 a	18.01 bc
NPDUD + 2	3.19 bc	1.82 c	4.41 bcd	nd	2.54 c	16.05 bcd
EPCDUD	3.51 b	nd	4.02 cde	3.05 b	5.89 ab	20.19 b
EPCDUD + 2	4.26 a	nd	5.14 abc	1.51 c	nd	15.31 bcd
EPODUD	2.78 c	3.55 a	2.65 ef	nd	nd	12.41 cd
EPODUD + 2	3.05 bc	3.08 ab	nd	2.88 b	7.10 a	16.11 bcd

nd = not detected; values within columns followed by the same letter are not significantly different according to Duncan Multiple Range Test ($P < 0.05$).

key flavour components such as sativin, as well as overall appearance quality traits.

3.3.3. PCA analysis

PCA extracted five components with Eigenvalues > 1.0, but the majority of variation was contained within PCs 1–3 (80.6%, cumulative). On this basis, only these three components were selected for presentation (Fig. 3a & b). The majority of explained variance was contained in PC1 (51.6%) and separates the majority of detected VOCs with the exceptions of DMDS, DMSO, and 1-butanol. Many of these compounds share significant correlations (Pearson *n*-1, $P < 0.001$), which are largely explained by their presence and higher abundances in temperature abused and late-stage shelf life samples. PC2 (18.4%) separates strongly for both DMSO and 1-butanol, and are highly associated with temperature abuse sample NPDUD. PC3 (10.7%) separated strongly for the GSL compounds diglucothiobeinin, glucosativin, DMB, and total GSLs. PC4, while containing minimal explained variance (7.2%) did separate both glucoraphanin and glucoerucin to a high degree.

Within the biplots (Fig. 3a & b) this information can be seen as a clear separation of low temperature, early shelf life samples, and samples that suffered prolonged temperature abuse, separating primarily along PC1. In Fig. 5a, NPDUD, NPDUD + 2, and TASDUD + 2 separate along PC2, with the latter time point associated with high abundances of alcohols (indicative of fermentative bacteria) and DMTS. The NPDUD samples by contrast are more associated with esters, ketones, aldehydes, furans, and DMSO.

By comparison, those samples that suffered little or no temperature abuse are tightly clustered to the left of both plots. These samples are associated with a higher concentration of glucosativin and DMDS, a precursor to DMS and DMSO. By maintaining cool-chain temperatures more effectively supermarkets would preserve key flavour compounds as well as prevent the degradation and diversity of disulfide compounds produced within bags. Fig. 3b shows that PC3 separates strongly for GSLs and DMDS, and reveals a slight separation between samples of little or no temperature abuse (Control, TAC4H, TAS4H, and EPCDUD) and those that suffered a more prolonged duration of shelf life (TACDUD, TACDUD + 2, and EPCDUD + 2). These latter time points separate slightly in the direction of glucoraphanin and more severe abuse temperature samples, indicating that some degree of temperature abuse may increase the abundance of glucoraphanin, and potentially of its ITC sulforaphane. The former time points are perhaps more associated with glucoerucin, another important GSL for health properties. The central position of these two compounds separating along PC3 perhaps indicates a separation of GSL profile according to storage conditions: Short-term temperature abuse and early sampling favors glucoerucin, whereas a certain degree of longer-term temperature abuse perhaps enhances glucoraphanin content to a degree.

Fig. 3c demonstrates that sulfides and other VOCs are perhaps indirectly related to the turnover of glucosativin. While not significant ($P < 0.05$) there is a distinct negative correlation between this GSL and sulfide compounds, as well as some alcohols. Glucosativin is a primary GSL of rocket and concentrations are highly dynamic over the course of shelf life (Bell et al., 2017). This dynamism is no doubt highly regulated, though the exact genetic mechanisms for its turnover are as yet unstudied. It is likely that this is closely linked with sulfur availability, but in our view it is unlikely that this compound is directly responsible for the production of DMS and other sulfides. The trend of sulfide production is far more in line with bacterial abundance, and this is in all likelihood the primary means by which off odors are produced within rocket salad bags. The two may be connected by changes in plant GSL and sulfur metabolism over the duration of shelf life, as well as protein and tissue breakdown. This clearly has implications for bacterial production of disulfides, perhaps through metabolism of S-methylcysteine sulfoxide (SMCSO), and a high bacterial load may accelerate such changes, especially if stored at an abusive temperature for long periods.

3.4. Bacterial abundance & gas composition of bagged fresh RTE wild rocket across two seasons during shelf life

3.4.1. Bacterial abundance

Total plate counts of fresh RTE rocket produced in different growing seasons varied significantly along the various points of post processing shelf life (Table 3). Rocket produced in summer contained significantly higher loads of bacteria than that produced in winter ($P < 0.0001$), as was hypothesised.

Rocket produced in summer and subjected to treatment TASDUD contained the highest bacterial loads, and this was followed by NPDUD, TASDUD + 2, and NPDUD + 2. The differences between them were not significant. Variations in handling and storage conditions of rocket in other treatments, including short temperature abuse (4 h) at 25 °C, did not significantly affect bacterial loads in leaves (TAS4H and TAC4H) compared to those in the control.

Among the treatments of winter rocket, leaves of treatment TASDUD + 2 contained the highest bacterial loads. Leaves treated under NPDUD, NPDUD + 2, and TASDUD + 2 were significantly different from the control sample but not between each other. The bacterial population of leaves in treatments TAS4H, TAC4H, TACDUD + 2, EPCDUD, EPCDUD + 2, EPODUD and EPODUD + 2 were generally low and did not differ significantly from the bacteria in the control leaves. The results were consistent with the previous experiment.

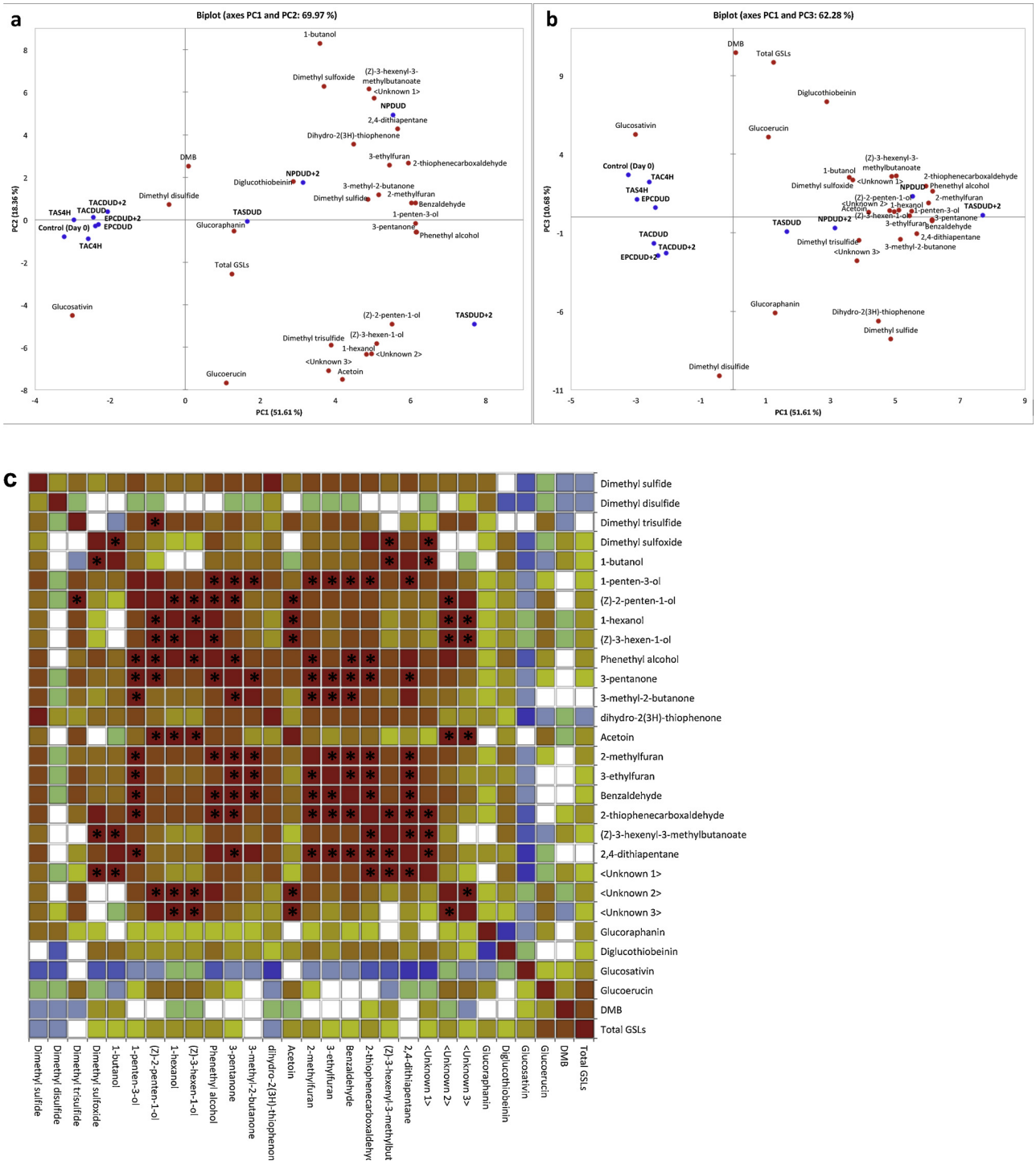

Fig. 3. Principal component analysis biplots of volatile organic chemical relative abundances and glucosinolate concentrations in differing temperature abuse and shelf life treatments of wild rocket, with Pearson *n*-1 correlation matrix. PC1 vs. PC2 (a) accounts for 69.97% of the explained variation. PC1 vs. PC3 (b) accounts for 62.28% of the explained variation. Blue circles with bold labels indicate scores values for each respective sample time point. Red circles indicate measured volatile and glucosinolate compounds. The correlation matrix (c) indicates the degree of correlation between compounds, ranging from red (positive), orange, yellow, white (no correlation), green, sky blue, and navy blue (negative). A star within a box indicates significance at $P < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.4.2. Gas composition

In both seasons, handling and storage treatments significantly affected the CO_2 and O_2 content of the bags ($P < 0.0001$; Table 3). Surprisingly, rocket grown in winter contained a higher percentage of CO_2, suggesting that the leaves have a higher respiration rate than summer leaves.

Among the summer samples, the gas in bags TASDUD (9.8%), TASDUD + 2 (9.9%), NPDUD (10.4%) and NPDUD + 2 (10.6%) contained a significantly higher concentration of CO_2 than the other treatments, suggesting that leaves with exposure to higher temperatures (25 °C abuse temperature and 13 °C during storage) have an increased respiration rate. TASDUD + 2 also contained the lowest O_2 content (2.47%; significantly different from all the other treatments), which supports this conclusion.

Table 3
Total plate counts, concentrations of carbon dioxide, oxygen, ethylene, and chlorophyll index of summer and winter grown rocket leaves subjected to varying handling and storage conditions post processing.

Treatment	Total plate counts (log cfu. g^{-1})		Carbon dioxide (%)		Oxygen (%)	
	Summer	Winter	Summer	Winter	Summer	Winter
Control	7.79 c	7.34 c	2.73 d	1.13 g	17.90 a	18.23 a
TAS4H	7.92 c	7.82 c	6.37 bc	7.36 e	13.07 b	12.20 c
TASDUD	10.17 a	7.34 b	9.77 a	14.17 a	7.87 c	0.33 h
TASDUD + 2	9.98 b	8.66 a	9.93 a	13.13 b	2.47 e	0.50 h
TAC4H	7.78 c	7.73 c	4.23 cd	4.74 f	16.27 a	14.70 b
TACDUD	8.58 c	8.04 bc	5.40 bc	8.56 d	13.94 b	9.30 e
TACDUD + 2	8.94 c	7.97 bc	5.70 bc	9.50 c	13.37 b	7.30 g
NPDUD	10.03 b	8.63 a	10.43 a	14.23 a	6.87 cd	0.33 h
NPDUD + 2	10.06 b	8.59 a	10.63 a	12.73 b	5.63 d	1.23 h
EPCDUD	8.33 c	7.94 bc	5.37 bc	7.73 e	13.47 b	10.47 d
EPCDUD + 2	8.70 c	7.97 bc	6.87 b	8.70 d	12.27 b	8.33 f
EPODUD	8.51 c	8.13 bc	na	na	na	na
EPODUD + 2	9.27 c	8.06 bc	na	na	na	na

na = not applicable as bags were opened; mean values within columns followed with the same letter are not significantly different according to Duncan Multiple Range Test ($P < 0.05$). All parameters between seasons were significantly different ($P < 0.0001$).

The gas sampled from the bags immediately after the arrival of the leaves at Day 0 contained the lowest percentage of CO_2 (2.73%), suggesting respiration rates are initially low. This is supported by the high O_2 percentage in the control (17.9%). This was followed by TAC4H (16.3%); both were significantly different from all other treatments.

Similar trends in the release of CO_2 were observed for winter rocket, whereby exposure to high temperatures led to a higher respiration rate in the bag, as seen in the TASDUD (14.2%), TASDUD + 2 (13.1%), NPDUD (14.2%) and NPDUD + 2 (12.7%) treatments. As for the summer grown rocket, the control samples had the lowest relative respiration rate (CO_2, 1.13%). O_2 was lowest at TASDUD (0.3%) and TASDUD + 2 (0.5%), indicating that mishandling by hypothetical supermarket retailers is potentially extremely detrimental to the shelf life of rocket by increasing respiration rates. O_2 was also lower in winter than in summer, suggesting a higher respiration rate in the former. Combined with the high bacterial loads of these samples in both seasons, and high concentrations of DMS observed at these time points in the previous experiment, it is evident that temperature abuse could negatively impact the quality of rocket.

4. Discussion

4.1. Temperature regulates microbial growth on wild rocket leaves during shelf life

The results presented in this study clearly show that temperature plays a critical role in controlling microbial numbers and are in agreement with previous studies conducted in rocket and other leafy vegetables (Carmichael et al., 1999; Luca et al., 2017; Martínez-Sánchez et al., 2006). As per the results and recommendations of previous studies (Khalil, 2016), leafy salads such as rocket should be kept constantly at < 5 °C throughout the supply chain and consumer shelf life to minimize microbial and pathogen propagation. This is evidenced throughout our study, where the lowest counts of bacteria were consistently observed in treatments where leaves were stored at a temperature of 4 °C. Where cool-chain temperatures were abused (≥13 °C) microbial growth increased significantly.

Different species of bacteria require different temperatures for optimum growth. Some are able to grow at temperatures even lower than those commonly used for the storage of fresh produce; however pathogenic strains are less likely to multiply and become established at these temperatures (Koseki and Isobe, 2005). Bacterial counts still increased in these treatments throughout shelf life storage, although by a much smaller degree than temperature abused samples.

Kou et al. (2015) demonstrated that temperature gradients within retail display cases significantly impact the product quality of baby spinach leaves. Leaves in bags at the front and back of display cases suffered the worst effects due to temperatures either being too high (front) or too low (back). The simple modification of installing insulating foam boards to the display cases reduced the temperature gradient significantly and maintained cool-chain temperatures (< 5 °C; Kou et al., 2015). Our data have shown that log-fold increases in bacterial loads are a direct result of sustained high temperature abuses, and this can be seen visually within products (Figs. S2 and S3). By preventing such temperature abuses within the store, this would in turn reduce the likelihood of food waste losses within the home due to the formation of off-odours or discoloration. Several studies have been published in recent years to assess the impact of modified atmospheres and improve shelf-life quality (Char et al., 2012; Inestroza-Lizardo et al., 2016). While these are interesting approaches, perhaps still the most simple and cost-effective way of reducing spoilage and food waste by microbial proliferation is to improve retail display temperatures and educate consumers on best practice for storage of leafy vegetable products such as rocket.

4.2. Adverse handling by the consumer

Rapid increases in bacterial counts were observed in open bag treatments (Table S2 and Table 3), which could be a potential source of pathogenic contamination during shelf life. Despite this, our results showed that when the rocket was put in contact with hands and subsequently stored again at low temperature (4 °C), this did not result in a significant increase in the number of bacteria, even if the rocket was kept for up to two days after the DUD (Table 3). A similar result was also observed for rocket that was exposed to high temperatures for 4 h, then kept at 4 °C, suggesting that even though temperature was abused the subsequent reintroduction and maintenance of cool-chain temperature retards the growth of the microbes. Disruption of the cold chain for a short period of time (4 h) can result in significantly higher bacterial growth if the cold chain is not subsequently maintained. Therefore, the shelf life, and possibly the health of consumers (if the bacteria happen to contain pathogenic strains) are at risk if supermarkets or consumers do not preserve the cool-chain more adequately.

There are various sources that may contribute to the contamination of rocket salad, and these may occur throughout the whole production chain. It may begin at the farm level; potential sources include water used for irrigation, and in the phases during production of the rocket salad, perhaps being introduced by workers. Incorrect irrigation and fertilization practice may contribute to the presence of pathogenic micro-organisms of human origin in minimally processed vegetables (Legnani and Leoni, 2004). Other factors include the use of manure as fertilizers, and the incorrect application of Good Manufacturing Practice (GMP) and the Hazard Analysis and Critical Control Point (HACCP) system during production, processing, packaging and distributions (De Giusti et al., 2010). This includes cross contamination from staff handling the products, and also from the processing equipment if not cleaned thoroughly. Although RTE vegetables are usually colonized by harmless saprophytic Gram-negative microflora, they are capable of affecting the shelf life of food products, and generate off-odours, such as sulfides (Luca et al., 2017).

4.3. Handling & storage conditions during shelf life affect the occurrence & abundance of volatile organic compounds

Key VOCs identified at later points of storage in this study were DMS and DMTS. These are potent inducers of off-odours in rocket and other Brassicaceae vegetables, and their generation is likely to adversely

affect consumer acceptance (Jacobsson et al., 2004). These compounds have been detected in previous shelf life studies of rocket salad (Bell et al., 2016).

DMS is an important compound found predominantly in cooked *Brassicas*, such as broccoli (Spadone et al., 2006). It has also been identified as a key odourant that produces sulfur aromas, but is linked with characteristic off-odours (Engel et al., 2002). Bacteria within salad bags, typically under anaerobic conditions, can produce DMS as oxygen is depleted within the atmosphere over time; and as DMS has also been reported to have a low odour threshold (Zinder and Brock, 1978); this makes prevention of its formation essential to maintain consumer acceptability. In combination with the presented bacterial count data, the abundance of DMS in late shelf life samples indicates a causal link between the two (Spadafora et al., 2016).

DMDS is usually associated with thermal degradation of SMCSO in *Brassica* and *Allium* species and creates characteristic sulfurous odours (Kubec et al., 1998). Nielsen et al. (2008) reported that abundance of DMS and DMDS was higher when rocket samples were kept at 8 °C than those kept at 4 °C, which is consistent with our data. DMTS is known for generating a strong, cabbage-like odour that is interpreted by consumers as off-smelling (Akpolat and Barringer, 2015). Supermarkets should therefore endeavor to preserve cold-chain temperatures throughout shelf life storage to limit the generation of this unpleasant smelling compound. DMSO by comparison typically has a weak garlic-like aroma, but can further breakdown into DMS to produce a stronger off-odour (Spadafora et al., 2016).

As hypothesised, the relative abundance of each compound was found to be associated with number of days of storage and temperature; but trends were different between individual compounds (Fig. 3). Similar results were reported by Luca et al. (2016), who found that the initial abundance of VOCs released by wild rocket were low, but overall abundance increased with the time of storage. Our data support the hypothesis that sulfides increase with microbial loads and abuse of temperature during shelf life storage, and are not the direct degradation products of GSLs or their hydrolysis products.

The significance of storage temperature in the generation of VOCs in rocket was also reported by Spadafora et al. (2016). The authors reported that aldehydes (2,4-hexadienal, (*E/Z*)-2-hexenal, and (*E/Z*)-3-hexenal) were positively correlated with temperature when the bouquet of rocket salad headspace was analysed after storage at 0, 5 and 10 °C. It was hypothesised that increasing temperature enhanced the activity of enzymes, thus accelerating metabolic processes, leading to the generation of more species of VOCs and increasing their relative abundances. (*Z*)-3-hexen-1-ol is linked with wounding response (D'Auria et al., 2007), however it was only present in samples that had undergone temperature abuse and were beyond the DUD. This suggests that wounding by processing is not a major contributor to VOC release in early shelf life and any damage to leaves may not be severe.

The high abundances of alcohols within temperature abused bags is indicative of severe fermentation, and is suggestive of anaerobic conditions within bags after long periods of storage (Deza-Durand and Petersen, 2014; Smyth et al., 1998). From a sensory perspective, alcohols have been shown to have a lower relative contribution to aroma of *Brassica* leaves (de Pinho et al., 2009) compared to sulfides and ITCs, for example.

4.4. Variations in bacterial load & gas composition are affected by storage & growing season

As hypothesised, we found that rocket produced in summer contained higher bacterial loads compared to winter (Table 3). The abundance of bacteria observed in summer months is also in line with a previously suggested hypothesis for the increases in food-borne illness, due to higher temperatures and greater consumption of salads. Bacteria are likely to propagate more under such conditions, as well as increase the likelihood of cool-chain breaks (Harris et al., 2003). Caponigro et al. (2010) previously reported that loads in several species of RTE salads (lettuce, rocket, spinach and lamb's lettuce), produced in summer and autumn, were higher than those produced in winter and spring. The intrinsic factors of commercial rocket production and processing, such as duration of transport, factory processing and bagging, are not routinely and holistically taken into account within the scientific literature. As such, our data represent the closest 'real world' values that have been reported to-date for commercially available wild rocket salad.

The variation in bacterial counts associated well with gas composition sampled from the headspace of bags. The significance of the gas composition in bagged fresh RTE produce, and its relationship with bacterial loads, VOCs and quality is well acknowledged. Generally, after long storage (especially at an abusive temperature) the production of O_2 will be reduced and at the same time CO_2 will be elevated (Deza-Durand and Petersen, 2014). In a previous study, two sulfides increased when rocket was inoculated with *Pseudomonas* spp. and *Xanthomonas* spp. and at oxygen levels below 1% (Spadafora et al., 2016). This level of O_2 was observed after storing rocket leaves at 10 °C for six days, and 5 °C for nine days by Spadafora et al. (2016), which occurred concurrently with increases in microbial counts. Our data support this observation (Table 3), as all instances of $O_2 < 1\%$ also corresponded to the highest TPCs for winter grown rocket (TASDUD, TASDUD + 2, and NPDUD). Therefore, temperature abuse during storage (13 °C) significantly impacts the respiration rate of rocket leaves, and also leads to sulfide formation through increased microbial loads.

Differences in growing environment markedly affected all gas composition measured. Summer grown rocket was found to have lower CO_2, but have higher O_2 in the headspace than winter rocket. High CO_2 in the winter bagged leaves could be attributable to high respiration, suggesting that the bacteria found on winter crops are acclimatized to lower temperatures, and might have poor adaptability to the (relatively) high abusive temperatures experienced during storage (Amodio et al., 2015). The data also indicate that the condition in the respective bags reached an anaerobic state during shelf life, thus potentially affecting the taste of leaves and generating of off-flavour volatiles (Deza-Durand and Petersen, 2014).

4.5. Handling & storage conditions affect the occurrence & abundance of glucosinolates

The impact of shelf life storage conditions upon health beneficial phytochemical precursors, such as GSLs, is not well studied or understood at the present time. Research by Bell et al. (2017) showed that GSLs and their hydrolysis products were significantly affected by processing and the supply chain; increasing in concentration relative to the point of harvest. The specific effects of MAP and the modification of bag atmosphere by leaves on GSLs in rocket are unknown. Leaf ontogeny is a key factor in determining the metabolic rate and speed of senescence (Koukounaras et al., 2007), and therefore GSL abundance is likely to be affected by these processes. Synthesis of GSLs and other secondary metabolites does not cease at the point of harvest, and hence will continue to change as a result of the conditions imposed by processing and shelf life storage practices (Jahangir et al., 2009; King and Morris, 1994).

Continuation of metabolic processes after harvest is reflected in the changes observed in rocket GSLs in this study, according to handling and storage conditions. In general, lower temperatures seem to preserve and maintain key GSL compounds, such as glucoerucin and glucosativin. This is important for both health benefits and flavour. Glucosativin seems to be the most dynamic temporally, and is in agreement with Bell et al. (2017). The study demonstrated this dynamism throughout processing and shelf life, and is likely a primary means by which rocket leaves respond to physical stress. Glucoraphanin, by comparison, is much more stable, in general, and may be indicative of differing biological functions of these molecules.

Microbial interactions with GSLs are intriguing, but are not well

understood. Bell et al. (2017) observed strong correlations between bacterial loads of leaves within the supply chain and GSLs concentrations. No causal link was established, but a recent publication has revealed that phyllospheric bacterial populations of *Eruca sativa* (arugula) and other Brassicaceae vegetables have a high degree of specificity to each species, as well as containing significant levels of myrosinase activity (Wassermann et al., 2017). This suggests that endemic bacteria are capable of metabolising GSLs, perhaps as a source of glucose (Hanschen et al., 2015) upon the leaf surface, and are resistant to the effects of ITCs, which have long been promulgated within the literature as anti-microbial compounds.

The influence of gas composition upon GSL biosynthesis within RTE rocket is also unknown. Anaerobic conditions that formed after prolonged storage coincided with the same time points as low concentrations of glucoerucin, for example, but the regulatory mechanisms for this specific response are yet to be elucidated. GSL biosynthesis is a complex metabolic pathway (Grubb and Abel, 2006), and a lack of available O_2 could potentially alter the types and abundances of specific GSLs. Martínez-Sánchez et al. (2006) observed that GSLs were reduced in wild rocket leaves by 60–100% when stored under low O_2 conditions, for example.

Our data show that even with detrimental effects induced by microbial loads and high temperatures, concentrations of glucoraphanin were not significantly affected. Indeed, concentrations for most treatments increased significantly relative to the control, indicating that even if maltreated, RTE rocket still likely contains significant amounts of precursor to the health beneficial compound SF. Previous studies have shown that glucoraphanin concentrations in rocket are not adversely affected during shelf life of rocket at 4 °C, and that SF is significantly enhanced over a seven day shelf life duration (Bell et al., 2017). Like erucin, SF is an important health beneficial compound of rocket (Manchali et al., 2012), and its preservation or enhancement through processing and shelf life practices should be a priority for breeders to select for. Glucoerucin, precursor to erucin, a potent anticancer compound (Melchini et al., 2009), was largely absent at high storage temperatures, which potentially reduces the nutritional quality of bagged RTE wild rocket. Maintaining cool-chain conditions within the supermarket would therefore aid in the preservation of important health benefits for the consumer. Overall, leaves that were kept at low temperature and sampled on the DUD (especially for open bags) contained the highest content of total GSLs. This was also observed for the leaves that were sampled two days after the DUD.

4.6. Conclusion

This study has highlighted that simulation of various temperatures, storage conditions and sampling scenarios of shelf life conditions produces significant and reproducible changes in microbial populations within processed RTE bags of wild rocket. This is the first study to analyse bacterial loads and changes from a perspective that accounts for conditions that may be experienced by a consumer and determine the effects of temperature abuse on VOC production, atmospheric composition, and GSL content. The data indicate that storing RTE rocket at 4 °C prevents significant microbial growth, which in turn reduces the amount of off-odour sulfide compounds and alcohols that are generated later in shelf life, and potentially preserves important phytochemicals, such as GSLs. The low O_2 content in samples with increased sulfide and alcohol emission is also indicative of anaerobic respiration, which may be due to additional fermentative processes within leaves. Consumption of rocket leaves as soon as possible after purchase is therefore advisable to the purchaser in order to avoid issues as a result of temperature abuses, but also to maximize the potential health benefits associated with rocket. Further research is required to understand bacterial propagation at the species level under the scenarios tested here, and to determine the effects of endogenous myrosinase-active strains upon the biosynthesis of GSLs, hydrolysis products, and sulfide compounds.

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Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2018.10.021.

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